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APPLICATION NO.	FILING DATE	FIRST NAMED INVENTOR	ATTORNEY DOCKET NO.	CONFIRMATION NO.
09/620,227	07/20/2000	Moshe Flugelman	20092	3020

22249 7590 07/25/2002

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EXAMINER
BARRETT, THOMAS C

ART UNIT	PAPER NUMBER
3738	11

DATE MAILED: 07/25/2002

Please find below and/or attached an Office communication concerning this application or proceeding.

restart rejection (3)

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2249 7590 06/20/2002

QUIMICA ORGANICA DE MEXICO,
S.A. DE C.V.
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1338 "G" STREET, S.E.
WASHINGTON, DC 20003

EXAMINER	
BARRETT, THOMAS C	
ART UNIT	PAPER NUMBER
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Office Action Summary	Application No.	Applicant(s)
	09/620,227	FLUGELMAN, MOSHE
	Examiner	Art Unit
	Thomas C. Barrett	3738

-- The MAILING DATE of this communication appears on the cover sheet with the correspondence address --

Period for Reply

A SHORTENED STATUTORY PERIOD FOR REPLY IS SET TO EXPIRE 3 MONTH(S) FROM THE MAILING DATE OF THIS COMMUNICATION.

- Extensions of time may be available under the provisions of 37 CFR 1.136(a). In no event, however, may a reply be timely filed after SIX (6) MONTHS from the mailing date of this communication.
- If the period for reply specified above is less than thirty (30) days, a reply within the statutory minimum of thirty (30) days will be considered timely.
- If NO period for reply is specified above, the maximum statutory period will apply and will expire SIX (6) MONTHS from the mailing date of this communication.
- Failure to reply within the set or extended period for reply will, by statute, cause the application to become ABANDONED (35 U.S.C. § 133).
- Any reply received by the Office later than three months after the mailing date of this communication, even if timely filed, may reduce any earned patent term adjustment. See 37 CFR 1.704(b).

Status

1)☒ Responsive to communication(s) filed on *10 April 2002* .

2a)☐ This action is **FINAL**. 2b)☒ This action is non-final.

3)☐ Since this application is in condition for allowance except for formal matters, prosecution as to the merits is closed in accordance with the practice under *Ex parte Quayle*, 1935 C.D. 11, 453 O.G. 213.

Disposition of Claims

4)☒ Claim(s) *1-55* is/are pending in the application.

4a) Of the above claim(s) *13-18 and 26-50* is/are withdrawn from consideration.

5)☐ Claim(s) _____ is/are allowed.

6)☒ Claim(s) *1-12, 19-25 and 51-55* is/are rejected.

7)☐ Claim(s) _____ is/are objected to.

8)☐ Claim(s) _____ are subject to restriction and/or election requirement.

Application Papers

9)☒ The specification is objected to by the Examiner.

10)☒ The drawing(s) filed on *20 July 2002* is/are: a)☐ accepted or b)☒ objected to by the Examiner.

Applicant may not request that any objection to the drawing(s) be held in abeyance. See 37 CFR 1.85(a).

11)☐ The proposed drawing correction filed on _____ is: a)☐ approved b)☐ disapproved by the Examiner.

If approved, corrected drawings are required in reply to this Office action.

12)☐ The oath or declaration is objected to by the Examiner.

Priority under 35 U.S.C. §§ 119 and 120

13)☐ Acknowledgment is made of a claim for foreign priority under 35 U.S.C. § 119(a)-(d) or (f).

a)☐ All b)☐ Some * c)☐ None of:

1.☐ Certified copies of the priority documents have been received.

2.☐ Certified copies of the priority documents have been received in Application No. _____ .

3.☐ Copies of the certified copies of the priority documents have been received in this National Stage application from the International Bureau (PCT Rule 17.2(a)).

* See the attached detailed Office action for a list of the certified copies not received.

14)☐ Acknowledgment is made of a claim for domestic priority under 35 U.S.C. § 119(e) (to a provisional application).

a) ☐ The translation of the foreign language provisional application has been received.

15)☐ Acknowledgment is made of a claim for domestic priority under 35 U.S.C. §§ 120 and/or 121.

Attachment(s)

1) ☒ Notice of References Cited (PTO-892)
2) ☐ Notice of Draftsperson's Patent Drawing Review (PTO-948)
3) ☐ Information Disclosure Statement(s) (PTO-1449) Paper No(s) _____ .
4) ☐ Interview Summary (PTO-413) Paper No(s). _____ .
5) ☐ Notice of Informal Patent Application (PTO-152)
6) ☐ Other:

DETAILED ACTION

Election/Restrictions

Applicant's election with traverse of Group I in Paper No. 5 is acknowledged. The traversal is on the ground(s) that the inventions of Group I and Group III are materially the same. This argument is found to be persuasive; therefore both Groups I and III will be examined.

Claims 13-18 and 26-50 are withdrawn from further consideration pursuant to 37 CFR 1.142(b), as being drawn to nonelected inventions, there being no allowable generic or linking claim. Applicant timely traversed the restriction (election) requirement in Paper No. 7.

Drawings

This application has been filed with informal drawings which are acceptable for examination purposes only. Formal drawings will be required when the application is allowed.

Color photographs and color drawings are acceptable only for examination purposes unless a petition filed under 37 CFR 1.84(a)(2) is granted permitting their use as acceptable drawings. In the event that applicant wishes to use the drawings currently on file as acceptable drawings, a petition must be filed for acceptance of the color photographs or color drawings as acceptable drawings. Any such petition must be accompanied by the appropriate fee set forth in 37 CFR 1.17(h), three sets of color drawings or color photographs, as appropriate, and an amendment to the first

paragraph of the brief description of the drawings section of the specification which states:

> The patent or application file contains at least one drawing executed in color. Copies of this patent or patent application publication with color drawing(s) will be provided by the U.S. Patent and Trademark Office upon request and payment of the necessary fee.

Color photographs will be accepted if the conditions for accepting color drawings have been satisfied.

Specification

The incorporation of essential material in the specification by reference to a foreign application or patent, or to a publication is improper. Applicant is required to amend the disclosure to include the material incorporated by reference. The amendment must be accompanied by an affidavit or declaration executed by the applicant, or a practitioner representing the applicant, stating that the amendatory material consists of the same material incorporated by reference in the referencing application. See *In re Hawkins*, 486 F.2d 569, 179 USPQ 157 (CCPA 1973); *In re Hawkins*, 486 F.2d 579, 179 USPQ 163 (CCPA 1973); and *In re Hawkins*, 486 F.2d 577, 179 USPQ 167 (CCPA 1973).

The lengthy specification has not been checked to the extent necessary to determine the presence of all possible minor errors. Applicant's cooperation is requested in correcting any errors of which applicant may become aware in the specification.

Claim Rejections - 35 USC § 112

The following is a quotation of the second paragraph of 35 U.S.C. 112:

> The specification shall conclude with one or more claims particularly pointing out and distinctly claiming the subject matter which the applicant regards as his invention.

Claims 1-12, 23 and 52-55 are rejected under 35 U.S.C. 112, second paragraph, as being indefinite for failing to particularly point out and distinctly claim the subject matter which applicant regards as the invention.

In claims 1 and 52, the use of the phrases "being coated" and "being genetically transformed" makes the claims unclear. The removal of the word "being" would make the claims less indefinite.

Regarding claim 23, it is unclear what the group consists of because of the word "and" before bone marrow".

Claim Rejections - 35 USC § 102

The following is a quotation of the appropriate paragraphs of 35 U.S.C. 102 that form the basis for the rejections under this section made in this Office action:

> A person shall be entitled to a patent unless –
>
> (b) the invention was patented or described in a printed publication in this or a foreign country or in public use or on sale in this country, more than one year prior to the date of application for patent in the United States.

Claims 1-5, 7-12, 19-22, 24-25, and 52–55 are rejected under 35 U.S.C. 102(b) as being anticipated by Pratt et al. (5,785,965). Pratt et al. discloses a prosthetic vascular ePTFE graft seeded with genetically transformed endothelial cells from the host or other sources (col. 3, lines 29-36).

Claims 1, 6, 19, 23 and 51 are rejected under 35 U.S.C. 102(b) as being anticipated by Huber et al. (Effects of Retroviral...) as cited in the Applicant's disclosure. Huber discloses a prosthetic vascular ePTFE graft seeded with transduced (genetically transformed) endothelial cells from a vein.

Please Note: The endothelial cells of the above prior art may not have been specifically transformed to express an adherence factor; however the cells do express adherence factors naturally and therefore meet the limitations. The Applicant discloses in the specification that the transformation causes an "*overexpression*" of adherence factors which the above prior art does not read upon. Also please be aware that the journal article "DANCE, a novel Secreted RGD..." cited by the Applicant in the disclosure, can be used for a 103 rejection with the above prior art if the Applicant only amends the claims to read "overexpress".

Conclusion

Any inquiry concerning this communication or earlier communications from the examiner should be directed to Thomas C. Barrett whose telephone number is (703) 308-8295. The examiner can normally be reached Tuesday-Friday between 9:00 A.M. and 6:00 P.M.

If attempts to reach the examiner by telephone are unsuccessful, the examiner's supervisor, Corrine McDermott can be reached on (703) 308-2111. The fax phone numbers for the organization where this application or proceeding is assigned are (703) 305-3580 for regular communications.

Any inquiry of a general nature or relating to the status of this application or proceeding should be directed to the receptionist whose telephone number is (703) 308 0850.

Thomas Barrett
June 17, 2002

CORRINE McDERMOTT
SUPERVISORY PATENT EXAMINER
TECHNOLOGY CENTER 3700

Notice of References Cited	Application/Control No. 09/620,227	Applicant(s)/Patent Under Reexamination FLUGELMAN, MOSHE	
	Examiner Thomas C. Barrett	Art Unit 3738	Page 1 of 1

U.S. PATENT DOCUMENTS

*		Document Number Country Code-Number-Kind Code	Date MM-YYYY	Name	Classification
	A	US-5,785,965	07-1998	Pratt et al.	424/93.1
	B	US-			
	C	US-			
	D	US-			
	E	US-			
	F	US-			
	G	US-			
	H	US-			
	I	US-			
	J	US-			
	K	US-			
	L	US-			
	M	US-			

FOREIGN PATENT DOCUMENTS

*		Document Number Country Code-Number-Kind Code	Date MM-YYYY	Country	Name	Classification
	N					
	O					
	P					
	Q					
	R					
	S					
	T					

NON-PATENT DOCUMENTS

*		Include as applicable: Author, Title Date, Publisher, Edition or Volume, Pertinent Pages)
	U	Huber et al. "Effects of -retroviral-mediated tissue plasminogen activator gene transfer and expression on adherence and proliferation of canine endothelial cells seeded onto expanded polytetrafluoroethylene" J Vasc Surg 1995;22:795-803.
	V	Nakamura et al. "DANCE, a novel secreted RGD protein expressed in developing, atherosclerotic and balloon injured arteries. JBC 1999;274,:22476-22483.
	W	
	X	

*A copy of this reference is not being furnished with this Office action. (See MPEP § 707.05(a).)
Dates in MM-YYYY format are publication dates. Classifications may be US or foreign.

Effects of retroviral-mediated tissue plasminogen activator gene transfer and expression on adherence and proliferation of canine endothelial cells seeded onto expanded polytetrafluoroethylene

Thomas S. Huber, MD, PhD, Theodore H. Welling, BS, Rajabrata Sarkar, MD, Louis M. Messina, MD, and James C. Stanley, MD, *Ann Arbor, Mich.*

Purpose: Seeding prosthetic arterial grafts with genetically modified endothelial cells (ECs) has the potential to substantially improve graft function. However, preliminary applications suggest that grafts seeded with retrovirally transduced ECs yield a significantly lower percent surface coverage than those seeded with nontransduced ECs. The objective of this study was to test the hypothesis that canine ECs transduced with the human tissue plasminogen activator (tPA) gene would have a lower rate of adherence to pretreated expanded polytetrafluoroethylene (ePTFE) both in vitro and in vivo and that they would proliferate at a slower rate on pretreated ePTFE in vitro.

Methods: Early passage ECs derived from canine external jugular vein were transduced with the retroviral MFG vector containing the gene for human tPA. ECs exposed to media alone served as controls. Iodine 125–labeled ECs were seeded in vitro onto ePTFE graft segments pretreated with canine whole blood, fibronectin (50 μg/ml), or media alone, and the percent of ECs adherent at 1 hour were determined ($n = 3$). Additional tPA-transduced and -nontransduced ECs were grown for 10 days on either fibronectin (50 μg/ml)–pretreated ePTFE wafers or tissue culture plastic pretreated with gelatin (1%) or fibronectin (50 μg/ml), and the EC proliferation rates were determined ($n = 3$). Furthermore, ^{125}I-labeled ECs were seeded onto fibronectin (50 μg/ml)–pretreated ePTFE graft segments implanted as carotid and femoral artery interposition grafts ($n = 3$). The grafts were harvested after 1 hour, and the percent of ECs adherent was determined.

Results: Human tPA was detected by immunohistochemical staining in 61% ± 5% of the transduced ECs and was expressed at 35.4 ± 12.9 ng/hr/10^6 cells. Fibronectin and whole blood pretreatment of the ePTFE grafts led to greater EC adherence in vitro than did media alone (90.9% ± 5.3% vs 77.8% ± 5.8% vs 4.7% ± 1.1%, $p \leq 0.05$). No significant difference in the rates of adherence or proliferation was seen in vitro between the transduced and nontransduced ECs. No significant difference in proliferation was found for the transduced ECs on the three matrices tested in vitro. In contrast, adherence of the transduced ECs in vivo was significantly lower than that of nontransduced ECs (64.7% ± 2.1% vs 73.7% ± 4.1%, $p \leq 0.05$) 1 hour after implantation.

Conclusions: Lower rates of surface endothelialization by genetically modified ECs in vivo do not appear to be due to an impaired capacity to initially adhere or proliferate on the synthetic graft but may result from decreased adherence after exposure to in vivo hemodynamic forces. (J VASC SURG 1995;22:795-803.)

From the Jobst Research Laboratories, Section of Vascular Surgery, Department of Surgery, The University of Michigan Medical School, Ann Arbor.

Reprint requests: Thomas S. Huber, MD, PhD, Section of Vascular Surgery, Department of Surgery, University of Florida, PO Box 100286, Gainesville, FL 32610-0286.

0741-5214/95/$5.00 + 0 24/1/66783

Use of small-caliber prosthetic bypass grafts has been limited by poor long-term patency rates. Endothelial cell (EC) seeding of these grafts has been proposed as a method to decrease their inherent thrombogenicity. Although animal studies have documented improved graft patency rates with this technique,[1,2] human applications have been inconsistent and have been largely abandoned.[3-5]

Recent advances in gene therapy have made it possible to seed prosthetic grafts with genetically modified ECs, thus offering considerable potential to reduce their inherent thrombogenicity by delivery of a desired gene product such as tissue plasminogen activator (tPA). The first application of this technology was reported by Wilson et al.,[6] who seeded Dacron grafts with retrovirally transduced ECs containing the lacZ marker gene and subsequently implanted them into the carotid artery position of dogs. Genetically modified ECs were identified on the lumen of the grafts 5 weeks after implantation. Podrazik et al.[7] seeded ECs retrovirally transduced with the lacZ gene onto expanded polytetrafluoroethylene (ePTFE) conduits that were implanted as thoracoabdominal grafts in dogs. A significantly lower percent surface endothelialization was detected at 6 weeks on grafts seeded with the transduced ECs compared with those seeded with the nontransduced, control ECs. Similarly, Sackman et al.[8] seeded ECs retrovirally transduced with a neomycin resistance gene onto ePTFE grafts implanted in dogs. Serial biopsies of graft surfaces revealed patchy EC coverage in the control (nontransduced) group at 18 weeks after implantation, whereas transduced ECs were detected only up to 3 weeks after implantation.

The studies of Podrazik et al.[7] and Sackman et al.[8] suggest that retrovirally transduced ECs seeded onto prosthetic grafts behave differently than nontransduced ECs after implantation of the grafts in vivo. Indeed, Brothers et al.[9] reported that canine ECs transduced with BAG retroviral vector containing the lacZ and Tn5 genes had a significantly lower proliferation rate during log phase growth in tissue culture. In contrast, Jaklitsch et al.[10] reported a comprehensive series of in vitro experiments examining cell attachment, proliferation, and migration of retroviral tPA–transduced bovine ECs. In these studies transduction led to a small decrease in the horizontal migration rate, but no differences in attachment or proliferation were observed.

This investigation was designed to characterize the function of tPA-transduced ECs on the surface of prosthetic grafts in preparation for a longer term in vivo study. Three specific hypotheses were tested: 1) canine jugular vein ECs retrovirally transduced to express the human tPA gene will exhibit a decreased initial adherence to pretreated ePTFE grafts in vitro; 2) allogenic canine jugular vein ECs retrovirally transduced to express the human tPA gene and seeded onto pretreated small caliber ePTFE grafts will exhibit a lower adherence rate 1 hour after implantation in vivo; 3) canine jugular vein ECs retrovirally transduced with the human tPA gene will proliferate at a slower rate than nontransduced ECs on the surfaces of pretreated ePTFE grafts in vitro.

MATERIAL AND METHODS

EC derivation and cultivation. ECs were harvested from excised external jugular veins of three adult male mongrel dogs with standard technique. The ECs were then resuspended in media (M199; Gibco Laboratories, Grand Island, N.Y.) supplemented with 20% bovine calf serum (Hyclone Laboratories, Logan, Utah), EC growth supplement (25 μg/ml, Collaborative Research, Inc., Bedford, Mass.), heparin (15 U/ml, Sigma Chemical Co., St. Louis, Mo.), glutamine (2 mmol/L, Gibco Laboratories), and penicillin/streptomycin (100 U/ml/100 μg/ml, Gibco Laboratories) and plated onto gelatin (1%, Sigma Chemical Co.)-coated 25 cm^2 tissue culture flasks (T25; Costar Corp., Cambridge, Mass.). The media was changed every 2 days until the ECs reached confluence. The concentration of the bovine calf serum in the media was decreased to 5% on the second day after harvest, with all other components of the media remaining the same (complete EC media). The confluent ECs were split 1:10 every 7 days. EC suspensions were obtained for both cell splits and cell counts with 0.1% trypsin/ethylenediamine tetraacetic acid (Gibco Laboratories), and EC counts were performed with a Coulter counter (Coulter Electronics, Inc., Hialeah, Fla.). The derived cells were confirmed to be more than 99% ECs by morphologic study and by demonstration of the ligand Dil-Ac–low-density lipoprotein (Biomedical Technologies, Inc., Stoughton, Mass.) with fluorescent microscopy.

Retroviral EC transduction. Optimal conditions for retroviral gene transfer with the MFG–human tPA vector have been previously reported from our laboratory.[12] The MFG-tPA vector was produced by the ΨCRIP producer line maintained in Dulbecco's modified Eagle medium–high glucose (Gibco Laboratories) supplemented with 10% fetal calf serum (Hyclone Laboratories) and penicillin/streptomycin (100 U/ml/100 μg/ml). Vector titers were determined by exposing subconfluent

Table I. Human tPA production (ng/10^6/hr)

Day	*Gelatin*	*Fibronectin*	*Fibronectin/ePTFE*
4	22.0 ± 6.5	25.7 ± 7.9	33.8 ± 2.6
10	27.2 ± 14.9	48.0 ± 7.9*	28.6 ± 17.7

*Significantly greater quantity of human tPA was produced on day 10 by the transduced ECs on fibronectin. Quantity of human tPA produced on days 4 and 10 by the transduced ECs in the proliferation study ($n = 3$) on the different matrix preparations (paired t test, $p \leq 0.05$). No significant difference was noted among the surface matrices for any given day (ANOVA/Scheffe's, $p \leq 0.05$).

plates of NIH 3T3 cells to serial dilutions of the vector stocks and performing immunohistochemical staining of these cells to identify clones expressing the human tPA. The vector supernatant was found to contain 0.5 to 1.5 × 10^6 colony forming units/ml and was found to be negative for the presence of any helper virus.[13] The ECs were plated on T25 flasks at a density of 5 × 10^3 cells/cm^2. Approximately 72 hours after plating during the log phase of growth, the ECs were exposed to the MFG-tPA vector in the presence of polybrene (8 μg/ml, Sigma Chemical Co.). Control cells were obtained by exposing the ECs to media alone (Dulbecco's modified Eagle medium–high glucose, 10% fetal calf serum, penicillin/streptomycin) in the presence of the polybrene. Two 12-hour exposures to either the vector or media were performed on subsequent days. ECs were used for the various protocols approximately 24 to 48 hours later.

tPA antigen assays. The quantity of human tPA secreted by transduced ECs was determined immediately before use of the cells in the various protocols. An aliquot of the media was collected from the T25 flasks, and the levels of the human tPA were measured with a modified enzyme-linked immunosorbent assay (ELISA) (Imubind-5; American Diagnostica, Inc., Greenwich, Conn.). The tPA secretion rate was expressed as nanograms/hour/10^6 cells.

Immunohistochemical staining. The transduced and nontransduced control ECs were plated onto gelatin-pretreated 12-well tissue culture plates (T-12; Costar Corp.) at the time of the graft seeding for the adherence studies and at the time of plating for the proliferation study. The ECs were allowed to adhere and spread for approximately 2 hours. They were then washed with phosphate-buffered saline solution (Gibco Laboratories), fixed in 3.7% formaldehyde (Eastman Kodak Co., Rochester, N.Y.) for 30 minutes, and permeabilized with 100% methanol (Fisher Scientific, Pittsburgh, Pa.) for 2 minutes. The cells were then sequentially incubated with 3% normal nonimmune rabbit serum (Vector Laboratories, Inc., Burlingame, Calif.) for 30 minutes and goat anti-human uteri tPA immunoglobulin G (50 μg/ml, American Diagnostica Inc.) for 2 hours. Subsequently, the ECs were exposed to biotinylated rabbit anti-goat IgG (5 μg/ml, Vector Laboratories, Inc.) for 30 minutes and incubated for 30 minutes with an avidin-biotin-horseradish-peroxidase complex (Vectastain ABC; Vector Laboratories, Inc.). Finally, the ECs were exposed to the peroxidase substrate 3-amino-9-ethylcarbazole (AEC; Vector Laboratories, Inc.) for 2 to 10 minutes. The stained EC monolayers were examined by a blinded observer, and the percentage of ECs staining positive for human tPA were determined.

EC labeling with ^{125}I. Confluent T-25 plates of ECs were incubated with 2 ml diluent C containing 6 μCi of ^{125}I-PKH-95 (Zynaxis Cell Science, Malvern, Pa.) for 1.5 minutes.[14] Bovine plasma–derived serum was then added for 1 minute. The plates were then washed five times with complete EC medium containing 20% bovine plasma–derived serum and refed with complete EC media containing 5% bovine plasma. ^{125}I-PKH-95 is a lipophilic compound that irreversibly binds to the EC membrane and does not leak into the surrounding tissues nor cells.[15-17] The radioactivity of the graft surfaces in the adherence studies was determined with a gamma counter (Packard Instrument Co., Meriden, Conn.).

In vitro EC adhesion. Early passage canine ECs (passage 2 to 4) from three separate animals ($n = 3$) were either transduced during log phase growth with the retroviral MFG-tPA vector or exposed to cell media alone. The ECs were labeled at confluence with ^{125}I and then seeded at a density of 10^4 cells/cm^2 for 60 minutes on 5 cm segments of 8 mm internal diameter, thin-walled ePTFE grafts. Graft surfaces were pretreated for 60 minutes immediately before EC seeding by exposure to one of three preparations: human fibronectin (50 μg/ml, Sigma Chemical Co.), canine whole blood, or cell media (M-199). The percent of adherent ECs were determined by the ratio of the radioactive counts on the graft surface to that of the seeding media. All experiments were performed in duplicate. EC expression of the human tPA gene was confirmed before graft seeding by ELISA

for the tPA in the media and by immunohistochemical staining.

EC proliferation. Early passage canine ECs (passage 2 to 4) from three separate animals ($n = 3$) were transduced during log phase growth with the retroviral MFG-tPA vector or exposed to cell media alone. At confluence, the ECs were passaged and plated onto either pretreated tissue culture polystyrene or pretreated thick-walled ePTFE wafers. The polystyrene was pretreated with either human fibronectin (50 μg/ml) or gelatin (1%) for 60 minutes. The ePTFE was pretreated with human fibronectin (50 μg/ml) for 60 minutes. Cell counts were determined 1, 4, 7, and 10 days after plating. Cell suspensions were prepared by enzymatically removing the ECs attached to the tissue culture plastic or ePTFE with 0.1% trypsin/ethylenediamine tetraacetic acid, and counts were determined by use of a Coulter counter. Complete EC removal was confirmed by microscopic evaluation of the tissue culture plastic or ePTFE. All experiments were performed in triplicate. EC gene expression of human tPA was confirmed before plating by an ELISA assay of the media and by immunohistochemical staining.

In vivo EC adhesion. Early passage canine ECs (passage 2 to 4) from three different animals ($n = 3$) were transduced during log phase growth with the retroviral MFG-tPA vector or exposed to cell media alone. ECs were labeled at confluence with ^{125}I and then seeded at a density of 10^4 cells/cm^2 for 60 minutes onto 8 cm segments of 4 mm internal diameter thin-walled ePTFE grafts. Graft surfaces were pretreated for 60 minutes immediately before EC seeding by exposure to human fibronectin (50 μg/ml). Transduced and nontransduced EC-seeded graft segments, 6 cm in length, were then implanted end-to-end in either the carotid or femoral artery positions of anesthetized (17 mg/kg Brevital, Eli Lilly Co., Indianapolis, Ind.) dogs in random order by use of a standard vascular technique. A total of four grafts were implanted in each animal with one transduced and one nontransduced EC-seeded graft implanted in each carotid and femoral artery position. Before implantation the animals were anticoagulated with heparin (150 μg/kg). After implantation, blood flow was restored, and the graft segments were perfused for 60 minutes, at which time they were harvested en toto by incision of the suture line and were gently washed to remove any nonadherent cells. Graft radioactivity was then determined. The percent of adherent ECs was determined by the ratio of the counts of the implanted graft surface to that of the nonimplanted graft segments that were seeded, incubated, washed, and counted in parallel. All experiments were performed in duplicate. EC gene expression for human tPA was confirmed before graft seeding by an ELISA assay of the media and by immunohistochemical staining.

Statistical analysis. The percent of transduced and nontransduced adherent ECs on graft surfaces both in vitro and in vivo were compared by use of a one-tailed, paired t test, with a p value ≤ 0.05 accepted as significant. The in vitro adherence of transduced ECs for the three graft pretreatments was compared by use of analysis of variance (ANOVA), and Scheffe's confidence intervals were used to determine the differences with a p value ≤ 0.05 accepted as significant. The best fit exponential curve was determined for the proliferation experiment and the doubling time calculated. The doubling times of both the transduced and nontransduced ECs were expressed as a percentage of the doubling time of the nontransduced ECs grown on gelatin in an attempt to decrease the variability between the individual, primary EC cultures. These normalized values for the transduced and nontransduced ECs were then compared by use of a one-tailed, paired t test, with a p value ≤ 0.05 accepted as significant. The normalized values for the transduced ECs on the three surfaces were compared by use of ANOVA, and Scheffe's confidence intervals were used to determine the significant differences with a p value ≤ 0.05 accepted as significant. The quantity of human tPA secreted by the transduced ECs in the proliferation protocol was compared between day 4 and day 10 by use of a two-tailed, paired t test, with a p value ≤ 0.05 accepted as significant. It was compared among surface pretreatments for a given day by use of ANOVA, and Scheffe's confidence intervals were used to determine the significant differences with a p value ≤ 0.05 accepted as significant. All statistical analyses were performed on a Macintosh Centris 610 (Apple Computer Corp., Cupertino, Calif.) computer with the Statview 512 software (Brain Power Inc., Calabasas, Calif.).

Experimental approval. All experimental procedures conformed to the guidelines established by the American Physiological Society and the National Institute of Health (*Guide for the Care and Use of Laboratory Animals*, NIH Publication No. 85-23, 1985) and was approved by the University of Michigan Committee on Use and Care of Animals.

Fig. 1. EC monolayer after immunohistochemical staining for human tPA. (Original magnification ×200.) Cytoplasm of ECs expressing human tPA stains *red*. Percentage of ECs expressing human tPA was determined by blinded observer.

The animals used for the in vivo adherence protocol were killed at the completion of the experiment with pentobarbital (Uthol; Butler Co., Columbus, Ohio).

RESULTS

High level expression of human tPA was detected in all ECs exposed to the MFG-tPA retroviral vector (35.4 + 12.9 ng/hr/10^6 cells). A high transduction efficiency was documented with human tPA detected by immunohistochemical staining in 61% ± 5% (mean ± SEM) of the ECs (Fig. 1).

Pretreatment of the graft surfaces with either fibronectin or blood led to significantly greater adherence for the transduced ECs relative to pretreatment with media alone (ANOVA/Scheffe's) (Fig. 2, *A*). No significant difference in adherence was detected between the transduced and nontransduced ECs, respectively, on the ePTFE graft segments treated with either fibronectin (99.8% ± 5.1% vs 90.9% ± 5.3%), blood (86.8% ± 3.2% vs 77.8% ± 5.8%) or media (4.8% ± 1.1% vs 4.7% ± 0.8%) (paired t test, $n = 3$) (Fig. 2, *B*).

No significant differences were noted among the normalized percent doubling times between the transduced and nontransduced ECs, respectively, on either the gelatin (114.4% ± 25.6% vs 100%), fibronectin (112.7% ± 14.2% vs 101% ± 28.4%), or ePTFE/fibronectin (166.5% ± 21.7% vs 123.6% ± 15.6%) surfaces (paired t test, $n = 3$), although a trend toward significance was noted in the latter group ($p = 0.15$) (Fig. 3). No significant difference was found for the doubling times of the transduced ECs on the three surface matrices (ANOVA/Scheffe's). High expression of the human tPA gene was detected by the transduced ECs on days 4 and 10 (Table I). A significantly greater quantity of human tPA was secreted by ECs seeded on fibronectin at 10 days (48.0 ± 7.9 vs 25.7 ± 7.9 ng/ml/10^6 cells/hr) (paired t test). There was no significant difference in the quantity of the human tPA among the surfaces for a given time point (ANOVA/Scheffe's).

In the in vivo experiment, both the transduced and nontransduced ECs adhered well to the fibronectin-pretreated ePTFE surface 1 hour after implantation and exposure to normal arterial perfusion (64% ± 2.1% vs 73.7% ± 4.1%), although transduced ECs adhered at a significantly lower rate (paired t test, $n = 3$) (Fig. 4).

DISCUSSION

This investigation provides several new contributions to our understanding of gene transfer to vascular cells. Retroviral transduction with the human tPA gene did not decrease the in vitro adherence of the ECs seeded onto pretreated ePTFE grafts. Human fibronectin and canine whole blood provided the best pretreatments for in vitro graft EC seeding. The proliferative rate of the transduced ECs was not

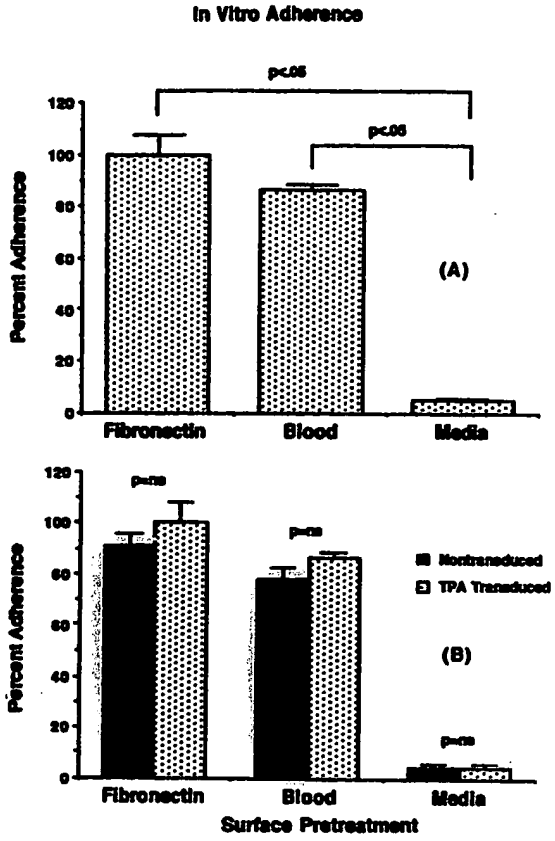

Fig. 2. In vitro adherence study. **(A)** Percent adherence of tPA-transduced ECs alone to ePTFE is shown for three surface pretreatments. Adherence of transduced ECs on ePTFE surfaces pretreated with fibronectin or blood were significantly greater than those pretreated with media alone (ANOVA/Scheffe's, $p \leq 0.05$). **(B)** Percent adherence of both tPA-transduced and -nontransduced ECs to ePTFE surfaces for three surface pretreatments ($n = 3$). No significant difference in adherence was seen between transduced and nontransduced ECs (paired t test, $p \leq 0.05$).

different from that of nontransduced ECs on gelatin, fibronectin, or fibronectin/ePTFE surfaces. Furthermore, no significant difference was seen in the proliferation rate of transduced ECs on the three matrices, and they continued to express high levels of human tPA throughout the duration of the experiment. Surprisingly, the adherence of tPA-transduced ECs on fibronectin-pretreated ePTFE grafts in vivo was significantly less than for the nontransduced ECs at 1 hour after implantation.

The mechanism responsible for the decreased adherence of the tPA transduced ECs in vivo is not known. Retroviral gene transfer and expression may lead to alterations in EC adhesion molecules either quantitatively or qualitatively and thus affect their ability to adapt to the shear stress of blood flow. Additionally, the human tPA expressed may exert a proteolytic effect on the underlying cellular matrix and lead to a sloughing of ECs from the graft surface. TPA acts by cleaving plasminogen to plasmin, and plasmin is known to induce proteolysis of matrix proteins such as laminin and fibronectin.[18] However, this explanation was partially refuted by Jaklitsch et al.,[10] who reported no decrease in adherence for tPA-transduced bovine ECs cultured on gelatin-treated tissue culture plates.

Transduced allogenic ECs seeded on the graft surfaces in this study could conceivably have induced an inflammatory or immune response. However, the significance of this is uncertain. Only hyperacute rejection caused by preformed circulating antibodies could explain rejection occurring within the 1-hour time course of the experiment. Furthermore, nontransduced allogenic ECs were included as controls. Yang et al.[19] recently reported that adenoviral vector–mediated gene transfer and expression is limited by low level viral genome expression, with the resultant viral-specific cell immunity leading to cell destruction. It is possible that retroviral transduction could lead to a similar response. Again, this type of response would not likely be contributory within the 1-hour time course of this study. Finally, the underlying biology of the retrovirus is such that it can exist as a stable entity within the host genome. Long-term gene expression has been reported within the vasculature by Lynch et al.[20] after seeding transduced smooth muscle cells onto injured rat carotid arteries. It is likely, however, that the biologic response of both the host and the transplanted ECs to seeding on a prosthetic graft differs from that of either ECs or smooth muscle cells seeded onto an injured or endarterectomized vessel. Krause et al.[21] have shown that ePTFE causes the activation of phagocytic leukocytes after adherence and is associated with an increased release of toxic oxygen intermediates. Additionally, Margiotta et al.[22] have shown that pretreating Dacron grafts with either fibronectin or whole blood increased the expression of intracellular adhesion molecule (ICAM-1) on seeded human saphenous vein ECs in vivo. ICAM-1 is a glycoprotein expressed on ECs that acts as a ligand for the lymphocyte-function-associated antigen that is responsible for cell-to-cell interaction in both the immune and inflammatory responses. It is possible that retroviral transduction may augment neutrophil-mediated EC destruction after seeding on

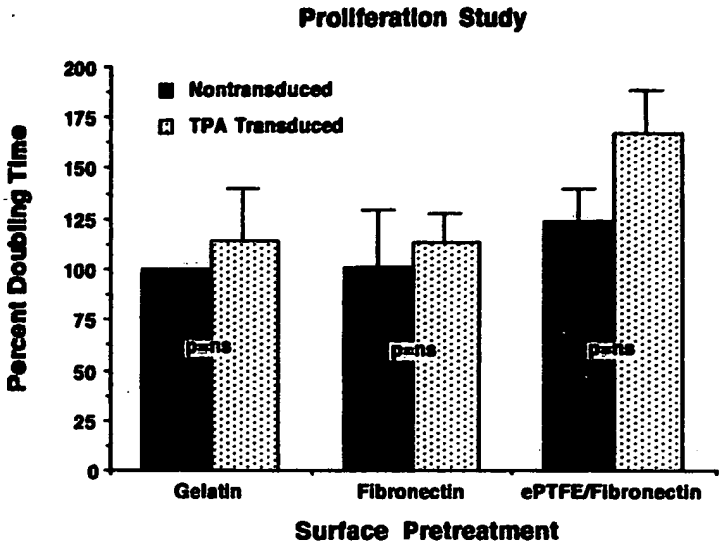

Fig. 3. Proliferation study. Normalized percent doubling times (doubling time expressed as percentage of nontransduced ECs grown on gelatin) of tPA-transduced and -nontransduced ECs for three surface pretreatments ($n = 3$). No significant difference was seen in doubling times between transduced and nontransduced ECs on any of prepared surfaces (paired t test, $p \leq 0.05$). Additionally, no significant difference in normalized percent doubling times for tPA transduced ECs was seen on three matrices (ANOVA/Scheffe's, $p \leq 0.05$)

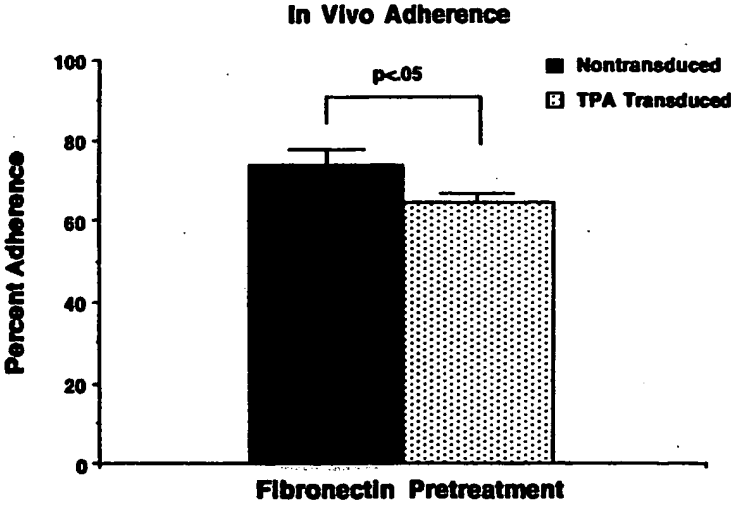

Fig. 4. In vivo adherence study. Percent adherence of both tPA-transduced and -nontransduced ECs to fibronectin-pretreated ePTFE surfaces is shown ($n = 3$) documenting that tPA transduced ECs had significantly lower adherence (paired t test, $p \leq 0.05$).

the prosthetic surface. Last, the human tPA gene product itself may induce an immune response in the dog, although many early efficacy studies of human tPA were performed without difficulty in the dog.[23-25]

The two in vitro components of this investigation provide both insight into the seeding of tPA-transduced ECs and optimism for application of this technique. Both whole blood and fibronectin pretreatment of the ePTFE graft surfaces resulted in greater than 78% EC adherence. Thus either preparation may be appropriate for in vivo applications.

The ePTFE graft surfaces were pretreated with fibronectin for the in vivo study because of the slightly greater adherence observed in our in vitro study, as well as reported by others.[26,27] The ability of the transduced ECs to proliferate on the various surfaces at comparable rates to nontransduced ECs while continuing to secrete tPA was likewise encouraging and concurs with the report of Jaklitsch et al.[10] Nevertheless, proliferation of ECs on fibronectin-pretreated ePTFE has not been a universal observation.[28] Clearly, the interpretation of the in vitro proliferation studies are limited because the growth conditions in vivo likely differ. Additionally, the surface of the grafts are modified in vivo by the deposition of fibrin and thrombus. Whole blood pretreatment of the ePTFE was not included in the proliferation study because of our initial in vitro adherence data and because of the practical reason that the blood and thrombus did not remain adherent to the ePTFE surfaces in tissue culture.

The MFG-tPA retroviral vector was chosen because of its efficacy and potential for human trials. Human tPA was expressed by 61.5% of the ECs exposed to the vector without the need for selection. Although selection, such as with the neomycin resistance gene, essentially assures a cell population of approximately 100% transduced cells, it adds to the complexity of the experiment and introduces potential for infection and contamination, thus limiting human applications of the technology. Furthermore, it has been suggested that the presence of the neomycin resistance gene and gene product may potentially inhibit proliferation.[7,9] However, it must be emphasized that our results are based on a cell population in which 61.5% of the cells exposed to the MFG-tPA vector expressed the human gene product. It is conceivable and possible that a cell population in which 100% of the cells expressed the human tPA would behave differently, but it is most likely that they would further emphasize the observed difference.

The experiment was designed such that nontransduced ECs served as the controls for the tPA-transduced ECs. These control cells were subjected to a sham transduction protocol, although they were not exposed to the retroviral vector. This control allows us to conclude that the observed decrease in adherence of the transduced ECs was due to the retroviral transduction and the human tPA gene expression. An appropriate additional control would have been ECs transduced with either the MFG vector containing a marker gene such as lacZ or a mock MFG vector containing all the necessary genes for viral incorporation and gene expression but lacking the tPA gene. Indeed, preliminary in vivo adherence and proliferation studies with the MFG-lacZ vector do not appear to be different than the tPA transduced and nontransduced ECs (data not shown).

This investigation supports the conclusion that the decreased percent surface endothelialization of prosthetic graft surfaces reported in the literature after seeding of retrovirally transduced ECs cannot be explained by either a decrease of EC adherence before graft implantation or a decrease in the proliferative rate of the transduced ECs on the graft surfaces. The reported decrease percent endothelialization may be partly explained by the impaired ability of the tPA-transduced ECs to adhere to the graft surface in vivo and suggests that the tPA retroviral transduction may alter the biologic function of the EC. This observation merits further investigation.

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DANCE, a Novel Secreted RGD Protein Expressed in Developing, Atherosclerotic, and Balloon-injured Arteries*

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ABSTRACT

We have identified and characterized mouse, rat, and human cDNAs that encode a novel secreted protein of 448 amino acids named DANCE (developmental arteries and neural crest epidermal growth factor (EGF)-like). DANCE contains six calcium-binding EGF-like domains, one of which includes an RGD motif. Overexpression studies of recombinant DANCE protein document that DANCE is a secreted 66-kDa protein. DANCE and recently described protein S1-5 comprise a new EGF-like protein family. The human DANCE gene was mapped at chromosome 14q32.1. DANCE mRNA is mainly expressed in heart, ovary, and colon in adult human tissues. Expression profile analysis by *in situ* hybridization revealed prominent DANCE expression in developing arteries. DANCE is also expressed in neural crest cell derivatives, endocardial cushion tissue, and several other mesenchymal tissues. In adult vessels, DANCE expression is largely diminished but is reinduced in balloon-injured vessels and atherosclerotic lesions, notably in intimal vascular smooth muscle cells and endothelial cells that lose their ability to proliferate in late stage of injury. DANCE protein was shown to promote adhesion of endothelial cells through

interaction of integrins and the RGD motif of DANCE. DANCE is thus a novel vascular ligand for integrin receptors and may play a role in vascular development and remodeling.

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Vascular development in the growing embryo requires the controlled proliferation of smooth muscle cells, endothelial cells, and fibroblasts and their continuous remodeling to form larger vessels. In most adult tissues, these vascular component cells are normally quiescent. However, vascular remodeling also plays an important role in many cardiovascular disorders (1). For example, in atherosclerosis and in restenosis after balloon angioplasty, vascular smooth muscle cells migrate and proliferate in the intima, resulting in the narrowing of the vascular lumen (2). In these conditions, cells are likely to reactivate fetal programs to enter the cell cycle. Indeed, the proliferating smooth muscle cells in the intima have changed from a contractile phenotype that can respond to vasoconstriction or vasodilation signals to a synthetic phenotype that can respond to growth stimulation. The synthetic phenotype may be regarded as an embryonic phenotype, as indicated by the switch in expression to an embryonic isoform of myosin heavy chain (3).

Extracellular matrix proteins are intimately involved in vascular remodeling, by affecting growth, migration, differentiation, and survival of vascular cell types (4). Integrins constitute a large family of cell surface receptors for extracellular matrix proteins that mediate not only cell adhesion by cell-matrix and cell-cell interaction but also multiple outside-in signals that lead to activation of downstream pathways such as tyrosine kinases and phosphatidylinositol 3-kinase (5). The most common integrin recognition sites of ligands contain a consensus Arg-Gly-Asp (RGD) motif. This RGD motif is recognized by many integrins ($\alpha_5\beta1$, $\alpha_{IIb}\beta_3$, and all $\alpha v\beta$ integrins), and synthetic peptides containing this RGD sequence are known to antagonize these integrins and inhibit angiogenesis or thrombosis (6).

Another important motif frequently seen in secreted proteins, including diverse vascular extracellular matrix proteins, is an epidermal growth factor (EGF)[1]-like motif (7). A subset of the EGF-like domains contain a distinctive amino acid sequence motif that is associated with calcium binding (8). This calcium-binding EGF-like (cbEGF) motif is found in several extracellular matrix proteins (fibrillin-1 and -2, fibulin-1 and -2, and nidogen) that are distributed in vessel walls, as well as in regulators of blood coagulation (factors IX and X, proteins C and S, and thrombomodulin), low density lipoprotein receptor, transforming growth factor-β-binding protein, Notch, and its ligands Delta and Serrate, which are known to be involved in cell differentiation. Tandemly repeated cbEGF domains in the presence of calcium ion form structurally stable helices that allow protein-protein interaction by these domains (9).

To isolate novel secreted molecules involved in the control of cardiovascular development and disease, we have employed the signal sequence trap method (10, 11), which is a newly developed cloning technique to isolate cDNAs of proteins containing signal sequences, including secreted, membrane, and endoplasmic reticulum proteins. We screened a cDNA library from mouse embryonic heart and isolated several new molecules. In this report, we describe the cloning and characterization of a novel secreted

protein with an RGD motif and cbEGF domains. This molecule, named DANCE (developmental arteries and neural crest EGF-like) protein after its embryonic distribution, is expressed mainly in developmental arteries and is re-expressed in atherosclerotic lesions and in balloon-injured arteries. DANCE promotes cell attachment via interaction of integrins and the RGD motif of the protein. DANCE is thus a new ligand of integrins that may play an important role in vascular remodeling.

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Signal Sequence Trap Screening, cDNA Cloning, and Northern Blot-- Poly(A) RNA from 9.5- and 18-dpc mouse embryonic heart was extracted with TRIzol reagent (Life Technologies, Inc.) and Oligotex-dT30 Super (Roche Molecular Biochemicals). The construction of the cDNA library and screening by yeast signal sequence trap was carried out as described previously (11). Full-length cDNAs were cloned by screening a mouse 13.5-dpc embryonic heart cDNA library (Stratagene). Two positive clones were sequenced. 5'-Rapid amplification of cDNA ends (RACE) was performed using the Marathon cDNA amplification kit (CLONTECH) according to the manufacturer's protocol, and six clones were sequenced. The rat DANCE homologue clone was obtained by screening a rat heart cDNA library (Stratagene) and by 5'-RACE. The human DANCE clone was obtained from IMAGE (GenBank^{TM} accession no. H17726). Each clone was sequenced and analyzed with the computer analysis program GeneWorks (IntelliGenetics, Inc.). A homology search was performed with BLAST and FASTA against public sequence data bases, and a motif search was performed on line at Prosite. Multiple sequence alignment was carried out using an on-line program.[2] For Northern blot analysis, a human multiple tissue Northern blot (CLONTECH) was hybridized to [^{32}P]dCTP-labeled full-length human DANCE cDNA (2.5 kilobase pairs) using QuickHyb (Stratagene). For Northern analysis of rat carotid arteries, RNA was extracted with TRIzol (Life Technologies, Inc.), and [^{32}P]dCTP-labeled full-length rat DANCE cDNA (2.5 kilobase pairs) was used as a probe. All blots were washed at 0.1× SSC, 65 °C, and exposed to autoradiography film overnight.

Chromosomal Mapping by Fluorescence in Situ Hybridization-- Full-length human DANCE cDNA was used as a probe. The probe was labeled by standard nick translation using biotin-16-dUTP (Roche Molecular Biochemicals) and was purified using Sephadex G-50 spin columns. Hybridization and detection by fluorescein isothiocyanate was carried out as described previously (12). Chromosomes were identified using counterstaining with 4',6'-diaminido-2-phenolindole dihydrochloride.

Protein Expression and Antibodies-- An *Eco*RI fragment including the full coding sequence of rat DANCE cDNA was cloned into a pCXN expression vector (gift from Dr. M. Kinoshita at Kyoto University), where expression is driven by the chick β-actin promoter and cytomegalovirus enhancer. Transfections of COS7 and 293T cells were performed using Lipofectamine Plus (Life Technologies, Inc.), according to the manufacturer's protocol. 16 h after transfection, the medium was changed to 8 ml of serum-free Dulbecco's modified Eagle's medium per 10-cm plate. 24 h later, the medium was harvested, and cells from 10-cm plates were lysed in 8 ml of radioimmune precipitation assay buffer (1× phosphate-buffered saline, 1% Nonidet P-40, 0.1% SDS). 25 μl of medium and cell lysate were analyzed

by SDS-polyacrylamide gel electrophoresis and immunoblotting. Rabbit anti-mouse/rat DANCE polyclonal antibodies were raised against KLH-conjugated polypeptide CMTRPIKGPRDIQLDLEMITVN, which corresponds to amino acids 406-426 of mouse and rat DANCE protein by TANA laboratories, LC. Western blotting was carried out following the ECL Western blot (Amersham Pharmacia Biotech) protocol.

In Situ Hybridization-- For *in situ* hybridization of mouse tissues, a *Xba*I-*Pst*I fragment (616 base pairs) was subcloned into pBlueScript in both directions. The sense and antisense RNA probe was transcribed *in vitro*. 7-μm paraffin sections from either mouse tissues or embryos were rehydrated, treated with 10 mg/ml proteinase K for 7.5 min, and hybridized with 10,000 cpm of ^{35}S-labeled riboprobe/ml in hybridization solution containing 50% formamide, 30 mM NaCl, 20 mM EDTA, 10 mM NaH_2PO_4, 10% dextran sulfate, 1× Denhardt's solution, and 10 mM dithiothreitol. Hybridization was carried out for 14 h at 60 °C. Samples were subsequently washed at 60 °C in 5× SSC, 10 mM dithiothreitol, 2× SSC, 50% formamide, 10 mM dithiothreitol; digested with RNase A (10 mg/ml) for 30 min at 37 °C; and dehydrated. Slides were dipped in Ilford-K5 photographic emulsion (Polysciences, Warrington, PA), exposed for 3 weeks, and developed in Kodak D19 solution. Counterstaining was performed in 0.02% toluidin blue/hematoxylin.

In situ hybridization of rat balloon-injured aortae was carried out on *en face* preparations of vessel segments as described (13), using [^{35}S]UTP-labeled sense and antisense rat DANCE riboprobes. In this model, endothelial regeneration and smooth muscle cell proliferation were studied in the thoracic aorta of which the endothelium had been removed with a 2F Fogarty balloon catheter. In this vessel, endothelial regrowth occurs from intercostal arteries, and rapidly proliferating smooth muscle cells migrate onto the denuded surface from the underlying tunica media starting at approximately 6-8 days after denudation. Once smooth muscle cells have migrated into the intima, they form an intimal lesion over the course of several weeks. By examining the cells on the luminal surface, this model allows the study of luminal smooth muscle cells at early times after injury, when cells are replicating, and at late times, when proliferation stops. Deendothelialized segments of arteries were identified by intravenous injection of Evans blue (0.3 ml of 5% solution in saline) 10 min prior to sacrifice. All animals were perfusion-fixed with phosphate (0.1 M, pH 7.4)-buffered 4% paraformaldehyde. For *en face* preparations, vessel segments were cut open longitudinally, and the tissue was pinned out flat on Teflon cards (luminal side facing up). Subsequent steps followed the protocol were as described (13).

Cell Attachment Assay and Attachment Inhibition Assay-- Human DANCE cDNA without the termination codon were subcloned to pEF6/V5 (Invitrogen) to add a C-terminal His_6 tag, and transfected to 293T cells. Recombinant protein was purified from the serum-free conditioned medium of stable lines, using nickel-nitrilotriacetic acid-agarose (Qiagen) according to established protocol, and dialyzed into Hanks' balanced salt solution containing 10 mM HEPES. Protein purity was confirmed by Coomassie Blue staining of SDS-polyacrylamide gel and Western blot. Protein concentration was determined by Coomassie Plus reagent (Pierce) using BSA as a standard.

Enzyme-linked immunosorbent assay 96-well plates (Nunc) were coated with 0.5-16 μg/ml of either DANCE protein or BSA diluted in Hanks' balanced salt solution for 18 h at 4 °C. The plates were

blocked for 1 h with a solution of heat-treated phosphate-buffered saline containing 10 mg/ml BSA. Human umbilical vein endothelial cells (HUVECs, from Clonetics) were harvested by trypsinization and resuspended in adhesion buffer (Hanks' balanced salt solution containing 10 mM HEPES, 2.2 mM $MgCl_2$, 0.2 mM $MnCl_2$, and 1% BSA). Cells (1.5×10^4/100μl) were added to each well in the presence or absence of peptides. Peptide antagonists included GRGDSP and control peptide GRGESP (both from Life Technologies, Inc.) at concentrations of 25-800 μM were preincubated with cells for 30 min before being placed in each well. Cells were incubated at 37 °C for 90 min, and wells were washed several times. 100 μl of medium was added to each well, and relative cell number was determined with the Cell Titer AQ reagent (Promega).

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Cloning and Sequencing of a Novel cDNA A55 (DANCE)-- We screened 6.6×10^6 yeast transformants from mouse 18-dpc embryonic heart and 6.0×10^6 yeast transformants from mouse 9.5-dpc embryonic heart, and 647 positive clones were obtained by the yeast signal sequence trap method, as described previously (14). All positive clones were sequenced, and redundant clones were removed. Of 62 independent clones, 33 were identical or homologous to sequences reported in mice or other mammals. One of the novel clones, A55, contained an EGF-like domain. Because the clones obtained by the screening contained only N-terminal sequence, poly(A)-tailed cDNAs for A55 were isolated from a murine 13.5-dpc embryonic heart cDNA library. 5' cDNAs containing the complete 5'-untranslated region were obtained by 5'-RACE. The sequence encodes a 448-amino acid protein (Fig. 1*A*). The translation start site methionine was assigned at nucleotide positions 320-322 because of the presence of an upstream stop codon (positions 266-268), N-terminal signal sequence, and the compatibility with a Kozak consensus sequence (15). The deduced amino acid sequence has an N-terminal hydrophobic domain, which is presumed to be the signal sequence and is predicted to be cleaved after Ala at position 23 but has no other hydrophobic regions that can serve as transmembrane domains and does not have a C-terminal endoplasmic reticulum retention signal (KDEL and related sequences), suggesting that A55 is a secreted protein. A55 was named as DANCE (developmental arteries and neural crest EGF-like) to represent its expression profile. Sequencing of the six clones from 5'-RACE revealed that only one contained a different 5'-untranslated region and N-terminal coding sequence (Fig. 1*A*). By sequencing a genomic clone containing the 5' region of DANCE, this sequence was found to exist just downstream of major exon 1 (data not shown).

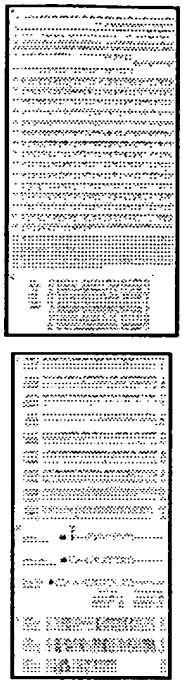

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Fig. 1. *A*, nucleotide sequence and deduced amino acid sequence of mouse A55 (DANCE) cDNA. Nucleotide numbers are based on the sequence of the major transcript, and *bracketed* nucleotide numbers indicate that of the minor, alternative spliced form of the transcript. Predicted signal sequence cleavage site using a program based on von Heijne's data (32) is shown by an *arrowhead*. An RGD motif found in the first cbEGF domain is *boxed*. Two putative Asn glycosylation sites are indicated by *asterisks*. *B*, alignment of six cbEGF domains of mouse DANCE with that of mouse EGF precursor (*EGF*), Notch, LDL receptor (*LDLR*), vitamin K-dependent protein S (*PRTS*), fibrillin 1 (*FBN1*), and fibulin-1 (*FBLN1*). *Numbers* indicate amino acid positions. Conserved residues including cysteines and those required for calcium binding are *shaded*. *C*, alignment of mouse, rat, and human DANCE amino acid sequences. For rat and human DANCE, only amino acid residues differing from those of the mouse clone are shown, and conserved amino acid residues are indicated by *dashes*. *D*, schematic diagram comparing DANCE, UPH1 (GenBank™ AF093119)/H411 (AF046870), and S1-5 (U03877)/T16 (D89730). N-terminal signal sequences are indicated as *black boxes*, and cbEGF domains are shown as *rounded rectangles*. The RGD motif, which is only found in DANCE, is also indicated. Amino acid identity of the cbEGF repeat and C-terminal domains between human clones of the family are shown *below*. *E*, alignment of the C-terminal domains of mouse DANCE and mouse fibulin-1C, fibulin-1D, and fibulin-2. Conserved amino acid residues that are identical to DANCE are *shaded*. *Numbers* indicate amino acid positions.

The DANCE transcript encodes a protein with six EGF-like domains (Fig. 1*B*). One is located at the N terminus, and the other five are tandemly distributed at the center of the molecule. These domains contain a consensus sequence associated with calcium binding: (D/N)*X*(D/N)(E/Q)X_m(D/N)*X_n(Y/F) (where *m* and *n* are variable and an asterisk indicates ß-hydroxylation) (16). This subset of the EGF domain with a calcium-binding signature has been classified as cbEGF domain and is well conserved among a variety of proteins (Fig. 1*B*). Tandemly repeated cbEGF domains form a rodlike helix structure, contributing to the structural integrity of the proteins. Not only important as structural elements, cbEGF domains are also known to mediate protein-protein interactions (17).

The deduced amino acid sequence of the DANCE transcript also contains two putative Asn glycosylation sites and an RGD motif in the first cbEGF domain, which is known to be a common ligand sequence motif of integrin ligands (Fig. 1*A*) (18).

Cloning of Human and Rat DANCE-- The rat homologue of DANCE was obtained by screening a rat heart cDNA library and by 5'-RACE. Partial sequence of a human DANCE homologue was found in EST data bases. By complete sequencing, this human clone turned out to contain the complete coding region. Alignment of mouse, rat, and human DANCE amino acid sequences reveals that DANCE is quite well

conserved among species (Fig. 1*C*). Amino acid identity between mouse and rat and between mouse and human is 98 and 94%, respectively. The RGD motif and putative Asn glycosylation sites are conserved among species.

DANCE, S1-5/T16, and UPH1/H411 Comprise a New EGF-like Protein Family-- A sequence homology search against the protein and nucleotide data base revealed that two proteins in particular are highly homologous to DANCE (Fig. 1*D*). S1-5 (human) was cloned from fibroblasts of a patient with Werner's syndrome and was reported to be overexpressed in Werner's syndrome and to stimulate DNA synthesis (19). T16 is a rat homologue of S1-5 and has 93% amino acid identity with S1-5. UPH1 (human) and H411 (Chinese hamster) are unpublished sequences found in the GenBank™ data base. UPH1 and H411 share 93% amino acid identity. All of these have six cbEGF domains, one at the N-terminal and five tandemly repeated in the middle of these molecules. These proteins have significant homology both in the cbEGF repeat (human DANCE *versus* UPH1, 54%; human DANCE *versus* S1-5, 48%; S1-5 *versus* UPH1, 57%) and C-terminal domain (human DANCE *versus* UPH1, 53%; human DANCE *versus* S1-5, 50%; S1-5 *versus* UPH1, 53%). Therefore, these proteins are considered to comprise a new EGF-like protein family, which we propose to call the "DUS" (DANCE, UPH1, and S1-5) family. Among the DUS family, only DANCE has an RGD motif in the first cbEGF domain.

C-terminal domains of DANCE and other DUS family members have a weak homology with those of fibulin-1C, fibulin-1D, and fibulin-2, which are extracellular matrix proteins with cbEGF domain repeats (20-22) but much larger than DUS family members (Fig. 1*E*). The C-terminal domain of fibulin-1C is reported to be involved in binding to nidogen (23), but function for those of fibulin-1D and fibulin-2 are not known. The multiple domain structure of DANCE protein suggests that it may bind to multiple receptors or ligands, *e.g.* integrin and extracellular matrix, or integrin and other cell surface receptors.

Recombinant Expression of DANCE Protein-- COS7 cells and 293T cells were transiently transfected with a full-length rat DANCE cDNA in an expression plasmid vector. Cell lysate and conditioned media of transfected cells were analyzed by Western blot using a polyclonal antibody raised against C-terminal polypeptide of rat DANCE (Fig. 2). DANCE protein was detected in both cell lysate and conditioned media, demonstrating that DANCE is a secreted protein. The size of the expressed DANCE protein is approximately 66 kDa. No dimer or trimer of DANCE was observed even with nonreducing gel electrophoresis and Western blot (data not shown).

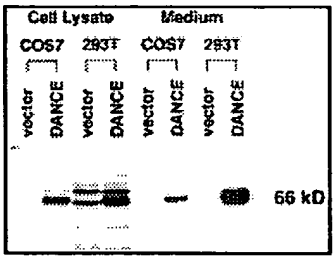

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Fig. 2. Recombinant protein expression of rat DANCE and detection by immunoblot. DANCE expression plasmid or vector plasmid were transfected into COS7 or 293T cells. After transfection, cells were cultured in serum-free media for 24 h. The media and cell lysate were analyzed by Western blot using anti-DANCE polyclonal antibody. Several artifactual bands that cross-reacted with anti-DANCE antibody are seen in 293T cell lysates.

Expression Profile of DANCE in Human Tissues and Chromosomal Mapping-- Northern blot analysis using poly(A) RNA from various human tissues and a full-length human DANCE cDNA probe revealed that the major transcript of 2.6 kilobase pairs is expressed mainly in heart, ovary, and colon (Fig. 3). Less expression is found in most of the tissues examined. However, it is undetectable in brain, liver, thymus, prostate, and peripheral blood leukocyte. This expression profile is largely different from that of S1-5, in which expression in heart is weak (24). Since each DUS family member is extremely conserved among species, it is likely that each member of the DUS family may have a distinct developmental or physiological role, despite the homology between the members.

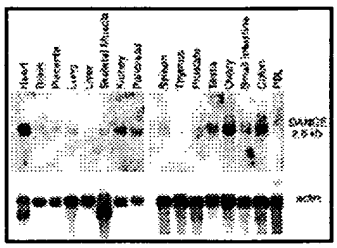

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Fig. 3. Northern blot analysis of human DANCE mRNA. The blots from various adult human tissues containing 2 μg of poly(A) RNA in each lane were probed with human DANCE cDNA. The *bottom panel* shows the same blot hybridized with β-actin. *PBL*, peripheral blood leukocytes.

The chromosomal localization of the human DANCE gene was determined by fluorescence *in situ* hybridization using full-length human DANCE cDNA as a probe. All 24 metaphase cells examined showed a specific hybridization signal with twin spots at 14q32.1 (Fig. 4, *A* and *B*). No known genetic disorder has thus far been mapped to this locus. The S1-5 locus is chromosome 2p16 (24), and fibulin-1 and fibulin-2 genes have been mapped on chromosomes 22q13.2-13.3 and 3p24-25, respectively (25, 26); thus, DUS family members and distantly related family genes have independent loci.

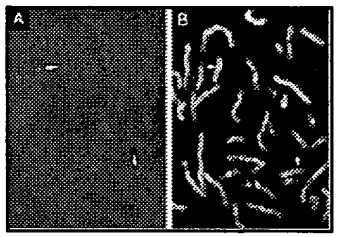

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Fig. 4. Chromosomal mapping of the human DANCE gene. Specific hybridization signals indicated by *arrows* are shown in *A*, which were identified as chromosome 14q32.1 with Q-banding of the same chromosomes in *B*.

DANCE Transcript Localization in Mouse Embryo, Adult Arteries, and Atherosclerotic Lesions-- DANCE expression in mouse development was studied by *in situ* hybridization. At 8.5 dpc, expression was restricted to endothelial cells of maternal placenta (Fig. 5, *A* and *A'*), whereas almost no signal was detected in embryonic tissues (data not shown). In 9.5-dpc embryos, migrating neural crest cells and the pericardium express DANCE (Fig. 5, *B* and *B'*). Branchial arch mesenchymal cells derived from neural

crest cells continue to express the DANCE gene. No expression is detected in the neural tube. At 12.5-dpc, the cardiac outflow tract and aorta display extremely strong expression, whereas less but significant expression is detected in several other tissues (Fig. 6, *A* and *A'*). In the heart, the DANCE transcript is present in endocardial cushion tissues, which derive from endocardial cells by an epithelial-mesenchymal transition (27) (Fig. 5, *C* and *C'*). Together with DANCE expression in neural crest cells, these observations suggest that one developmental function of DANCE may be to play a role in epithelial-mesenchymal transitions. Head mesenchyme, intersomitic tissues, and several other mesenchymal tissues also express DANCE (Fig. 6). In the aorta, DANCE expression is seen both in endothelial cells and in smooth muscle cells. In 14.5-dpc embryos, some neural crest-derived tissues such as head mesenchyme, cardiac outflow tract, and sympathetic ganglia continue to express DANCE, but some neural crest tissues such as adrenal gland do not (data not shown). Mesenchymal tissues in proximity to developing cartilages also express DANCE mRNA (data not shown). The aorta still exhibits the strongest expression of DANCE at 14.5 dpc.

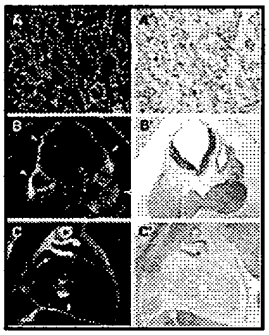

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Fig. 5. Expression of DANCE mRNA in several representative mouse embryonic sections as shown by *in situ* hybridization, viewed in dark field (*A*, *B*, and *C*) and bright field (*A'*, *B'*, *C'*). *A* and *A'*, high magnification view of maternal placenta at stage 8.5 dpc. DANCE expression is found in endothelial cells. *B* and *B'*, transverse section of rostral region of 9.5-dpc embryo. Neural crest cells migrating to branchial arches are indicated by *arrowheads*. Pericardium is indicated by an *open arrowhead*. *C* and *C'*, heart region of 12.5-dpc embryo. DANCE expression in outflow tract (*arrow*) and endocardial cushion tissue (*arrowhead*) are shown. Magnification: × 200 (*A* and *A'*), × 40 (*B* and *B'*), × 40 (*C* and *C'*).

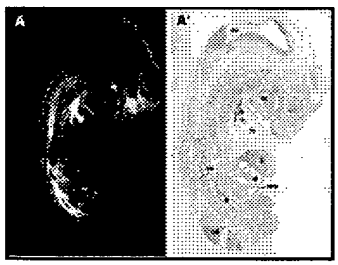

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Fig. 6. Expression of DANCE mRNA in a 12.5-dpc embryo as shown by *in situ* hybridization with dark field (*A*) and bright field (*A'*) views. *a*, aorta; *d*, duodenum; *h*, heart; *li*, liver; *m*, mandible; *mv*, mesenteric vessels; *nt*, neural tube; *o*, cardiac outflow tract; *tv*, third ventricle; *v*, vertebra. Magnification, × 40.

In adult aorta, DANCE expression is largely diminished. However, intense focal expression is found at intercostal branching points in the thoracic aorta (Fig. 7, *A* and *A'*). This observation is interesting in relevance to atherosclerosis, because alternation of hemodynamic stress at branching regions has been pointed out to induce atherogenesis at these regions (28, 29).

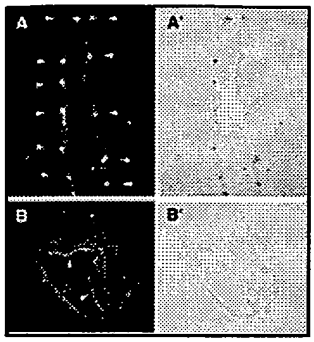

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Fig. 7. Expression of DANCE mRNA in normal adult mouse aorta and in atherosclerosis lesions of LDL receptor-deficient mice. Longitudinal section of normal adult thoracic aorta (*A* and *A'*) shows robust expression only in the intercostal branching points (*arrowheads*). Transverse section of the thoracic aorta of LDL receptor-deficient mice (*B* and *B'*) shows re-expression of DANCE mRNA in the endothelial cells overlaying fibrous plaques of atherosclerotic lesion (*arrowheads*), whereas only little expression is seen in the normal region (*open arrowhead*). Original magnification, × 40 (*A* and *A'*), × 40 (*B* and *B'*).

Accordingly, DANCE expression was studied in atherosclerotic vessels using LDL receptor-deficient mice fed with a high cholesterol diet (30). Endothelial cells overlying the plaques exhibited a significant increase in DANCE mRNA expression (Fig. 7, *B* and *B'*, *arrowhead*) compared with normal regions of the same vessel (*open arrowhead*).

Augmented Expression of DANCE in Balloon-injured Vessels-- To examine if DANCE is re-expressed in other settings of pathological vascular remodeling, DANCE transcript expression in balloon-injured rat carotid arteries and aortae was studied (31). Northern blot analysis revealed that DANCE mRNA expression was markedly increased following balloon injury with the highest levels seen at 14 days, coinciding with decreasing smooth muscle cell replication (Fig. 8). By *in situ* hybridization carried out with *en face* preparations of vessel segments, augmented expression of DANCE mRNA is observed in both endothelial cells and smooth muscle cells (Fig. 9). Endothelium from normal aorta shows no detectable expression (Fig. 9*A*). 8 days following endothelial wounding (Fig. 9*B*), little expression is seen at the leading edge of the regenerating endothelium (*dotted line*), but increased expression is evident further behind the leading edge where cells return to quiescence. Proliferating smooth muscle cells that migrated from the underlying media onto the luminal surface of the denuded aorta at 8 days after injury express DANCE mRNA (Fig. 9*D*), but even higher levels of expression are seen at 14 days when smooth muscle replication is decreasing (Fig. 9*E*). This observation suggests that DANCE may affect cell growth as a "brake" in autocrine or paracrine manner when proliferation should stop.

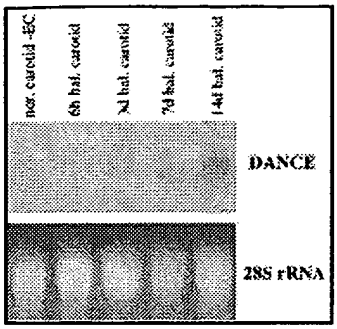

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Fig. 8. Expression of DANCE mRNA in the rat carotid artery following balloon catheter injury. The left carotid artery was denuded with a 2F balloon catheter as described (31). Total RNA was isolated from carotid arteries 6 h and 3, 7, and 14 days after balloon injury and from normal vessels with the endothelium removed (*nor. carotid -EC*) (33). RNA was analyzed by Northern blotting using DANCE cDNA as probe. An RNA loading control with ethidium bromide-stained 28 S rRNA is shown *below*.

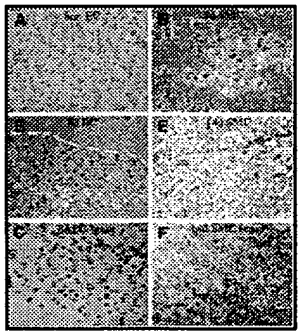

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Fig. 9. Expression of DANCE mRNA in endothelial cells and smooth muscle cells of injured rat aortae. *In situ* hybridization was carried out on *en face* preparations of vessel segments as recently described (13) using DANCE sense (*C* and *F*) and antisense probes (*A*, *B*, *D*, and *E*). *A*, normal endothelial cells. *B* and *C*, endothelial cells 8 days after denudation. The leading edge of regenerating endothelium is indicated by the *dotted line* in *B*. Proliferating smooth muscle cells that migrated to intima at 8 days after injury (*D*) and 14 days after injury (*E* and *F*). Magnification, × 400.

DANCE Protein Promotes Endothelial Cell Attachment through RGD-Integrin Interaction-- Because DANCE has an RGD motif, which is known as an integrin-binding motif, we studied whether it has a role in cell attachment. For this purpose, recombinant human DANCE protein was purified from the conditioned medium of transfected 293T cells. HUVECs spread on DANCE coated microtiter plates (Fig. 10*A*), whereas no cells spread on BSA-coated plates (Fig. 10*B*). This attachment to DANCE protein was dose-dependent (Fig. 10*C*). A synthetic peptide GRGDSP completely inhibited spreading and attachment of HUVECs to DANCE, whereas the control peptide GRGESP showed no inhibition (Fig. 10*D*). This RGD dependence of the cell attachment activity of DANCE suggests that the attachment is mediated by binding of cell surface integrins to DANCE protein.

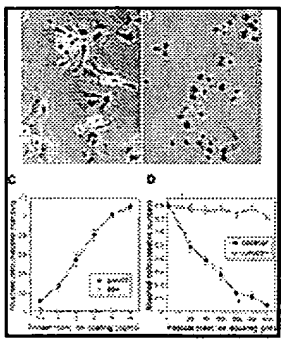

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Fig. 10. DANCE mediates adhesion of endothelial cells through binding to integrins. HUVECs were allowed to attach on DANCE-coated wells (*A*) or BSA-coated wells (*B*). Each well was coated with 8 μg/ml of respective protein solution. *C*, HUVECs were incubated on 96-well plates that were precoated with various concentrations of DANCE protein or BSA protein solution. After washing, the relative number of attached cells were determined using wells without washing as standards. Data were obtained as quadruplicate, and mean ± S.D. values are shown. *D*, HUVECs preincubated with various concentrations of GRGDSP or GRGESP peptides were allowed to attach to wells coated with 8 μg/ml DANCE. Wells were washed as above, and the relative numbers of attached cells were determined.

Considering the multiple domain structure and robust expression in embryonic arteries, atherosclerotic lesions, and balloon-injured arteries, an intriguing possibility is that DANCE may contribute to vascular remodeling via interaction with integrins and other extracellular molecules.

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FOOTNOTES

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The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EMBL Data Bank with accession number(s) AF112151 (mouse DANCE), AF112152 (rat DANCE), and AF112153 (human DANCE).

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[e] A National Institutes of Health Individual National Research Service Award of National Institutes of Health recipient.

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[2] This program is available on the World Wide Web.

ABBREVIATIONS

The abbreviations used are: EGF, epidermal growth factor; cbEGF, calcium-binding EGF-like; RACE, rapid amplification of cDNA ends; BSA, bovine serum albumin; HUVEC, human umbilical vein endothelial cell; dpc, day(s) postcoitum.

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